2-2010

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Applied and Environmental Microbiology, Feb. 2010, p. 985–987 Vol. 76, No. 3
0099-2240/10/$12.00 doi:10.1128/AEM.02172-09

Use of *rpsL* as a Counterselectable Marker in *Borrelia burgdorferi*▽

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Received 8 September 2009/Accepted 24 November 2009

We have demonstrated that *rpsL*, encoding the S12 protein of the small ribosomal subunit, can be used as a counterselectable marker in *Borrelia burgdorferi*, the causative agent of Lyme disease. Mutations in *rpsL* confer streptomycin resistance. Streptomycin susceptibility is dominant in an *rpsL* merodiploid, and streptomycin selects for the loss of wild-type *rpsL* carried in *trans*. This is the first description of a counterselectable marker in *B. burgdorferi*.

The approach for genetically manipulating *Borrelia burgdorferi*, a spirochete that causes Lyme borreliosis (2, 6, 23), has slowly matured over the past 15 years (17, 18). A number of selectable markers conferring antibiotic resistance have been developed and used to disrupt genes and maintain plasmids, including *gyrB* (Cou^r) (21), *aphI* (Kan^r) (5), *ermC* (Erm^r) (22), *aadA* (Spt/Str^r) (9), and *aacC1* (Gen^r) (8), which have enabled effective, if not efficient, molecular genetic tools. However, to date, no counterselectable marker has been available to select for the loss of a particular DNA sequence (16). We now demonstrate that susceptibility to streptomycin is dominant in a merodiploid carrying a wild-type *rpsL* allele and a streptomycin-resistant *rpsL* allele and that *rpsL* can function as a counterselectable marker in *B. burgdorferi*. This now provides a genetic tool, previously unavailable, for studying the biology of *B. burgdorferi* and the pathogenesis of Lyme disease.

In *Escherichia coli*, the *rpsL* gene encodes the S12 ribosomal protein of the 30S subunit. Streptomycin exerts its antimicrobial activity by binding to 16S rRNA, near the binding interface of S12, to inhibit protein synthesis by increasing translational errors through the recruitment of incorrect tRNAs (11, 12). Mutations in *rpsL* confer resistance in numerous bacteria, including *E. coli* (4, 10, 14) and the spirochete *Leptospira biflexa* (15). Streptomycin inhibits the growth of streptomycin-resistant *rpsL* mutants when wild-type (streptomycin-sensitive) *rpsL* is expressed in *trans*, indicating that the antibiotic susceptibility phenotype is dominant (13). Furthermore, growing merodiploid *rpsL* strains in the presence of streptomycin can select for the loss of wild-type *rpsL*, demonstrating the utility of this gene as a counterselectable marker (16).

We recently isolated *B. burgdorferi* mutants that were 10-fold more resistant to streptomycin than the parental strain, B31-A (7). One isolate contained a mutation in *rpsL* encoding the single amino acid substitution K88E in S12, the same residue that was found to be mutated in streptomycin-resistant *L. biflexa* (15) and *E. coli* (10). We constructed an *rpsL* merodiploid strain to test the feasibility of streptomycin susceptibility as a counterselectable marker in *B. burgdorferi*.

Construction of a counterselectable marker. *rpsL* was amplified from *B. burgdorferi* strain B31-A3 genomic DNA by PCR using the primers rpsLU133F+SgrAI (5′-CGCCGGTGACTGGAACTGGTATGGGTC-3′) and rpsL375R+SgrAI (5′-CACCGGCGTTAAGCTTTAGGCTTTTTTGTTC-3′). The PCR product was cloned into pCR2.1-TOPO (Invitrogen) to yield pTA*rpsL* and confirmed by direct sequencing. pTA*rpsL* was digested with XhoI and SacI and ligated into the *Borrelia* shuttle vector pBSV2 (24), which had been digested with SalI and SacI, to yield pBS*rpsL* (Fig. 1A). Low-passage B31-A was transformed with pBS*rpsL*, and the streptomycin-resistant strain DCSmR4 (7) was transformed with either pBS*rpsL* or the empty vector pBSV2, essentially as described previously (19). Cultures of transformants were diluted with Barbour-Stoenner-Kelly (BSK) II medium (1) containing kanamycin (200 μg/ml) in 96-well plates (25) to select for those containing pBSV2 or pBS*rpsL* harboring the kanamycin resistance gene *aphI*. Positive wells were screened for the presence of the plasmids by PCR using the primers PflgB5+MfeI (5′-CAATTGTACCCGAGCTTCAAGGAA-3′) and KanR 488R (5′-TCACTCGCATCAACCAAACC-3′) to detect *aphI*. Clones were chosen from 96-well plates that had fewer than 10 positive wells because the probability that a well was inoculated with a single cell is greater than 0.94 (J. M. Graham and D. S. Samuels, unpublished data).

Phenotype of *rpsL* merodiploid. We first tested the hypothesis that streptomycin susceptibility is dominant in an *rpsL* merodiploid. Each strain was initially grown in liquid BSK II medium containing only kanamycin to maintain pBSV2 or pBS*rpsL*. *Borrelia* spirochetes were then counted using a Petroff-Hausser counting chamber, and 1,000 spirochetes were plated in semisolid BSK medium (19) containing (i) no antibiotics, (ii) kanamycin (200 μg/ml), (iii) streptomycin (50 μg/ml), or (iv) kanamycin plus streptomycin. Plates were incubated at 37°C and 5% CO_2 for 2 weeks before enumeration. The merodiploid strain DCSmR/pBS*rpsL* did not form colonies in the presence of both kanamycin and streptomycin, suggesting that streptomycin susceptibility is dominant (Table 1). A few DCSmR/pBS*rpsL* colonies formed in the presence of streptomycin alone (Table 1). Similar results were confirmed using a second DCSmR/pBS*rpsL* clone (data not shown). The frequency of loss of pBS*rpsL* (0.04%) is much lower than we

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▽ Published ahead of print on 4 December 2009.

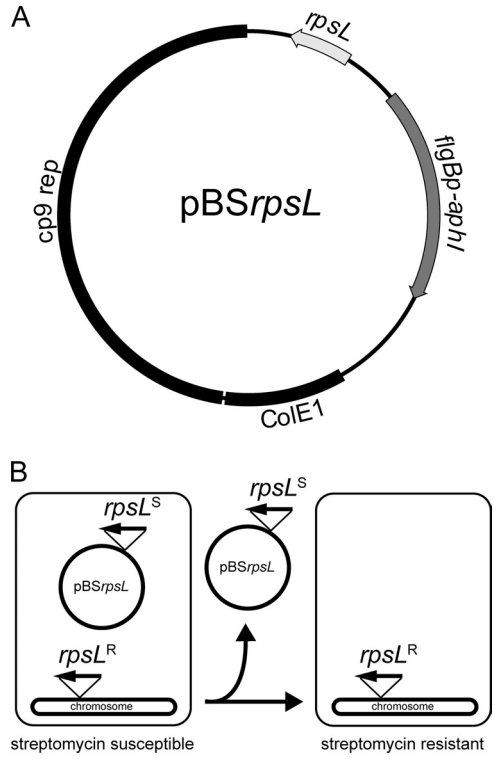

FIG. 1. Counterselection using *rpsL*. (A) Schematic of pBS*rpsL*. pBS*rpsL* was derived from pBSV2 by inserting the *rpsL* gene and its native promoter into the multiple cloning site. The plasmid also carries the kanamycin resistance open reading frame *aphI* under the control of the *flgB* promoter (*flgBp*) as well as an *E. coli* replication origin (ColE1) from pCR-XL-TOPO and a *B. burgdorferi* replication origin (cp9 rep) from the 9-kb circular plasmid cp9 (which includes genes *bbc01*, *bbc02*, and *bbc03* plus two inverted repeats). (B) Model of counterselection. The DCSmR4 spirochetes (rounded rectangles) carry a mutant *rpsL* (encoding a K88E mutation in S12) on the chromosome that confers resistance to streptomycin (*rpsL*^{R}). The plasmid pBS*rpsL* carries the wild-type *rpsL* gene driven by its own promoter, which confers susceptibility to streptomycin (*rpsL*^{S}). *B. burgdorferi* DCSmR4 cells that have lost the pBS*rpsL* plasmid can be selected for with streptomycin.

have observed with another counterselectable marker system using fluoroquinolones and *parC* (~3%) (18); the higher rate of plasmid loss in the *parC* mutants may be due to a partitioning defect. However, the pBSV2 backbone vector is stable, with 100% retention after ~90 generations without selection (24).

Counterselection with *rpsL*. Streptomycin should select for streptomycin-resistant *Borrelia* spirochetes that have lost pBS*rpsL*, which confers susceptibility, and that carry only the genomic *rpsL* allele conferring streptomycin resistance (Fig. 1B). To confirm that kanamycin-resistant colonies retained pBS*rpsL* and streptomycin-resistant colonies lost pBS*rpsL*, DNA from individual colonies was analyzed by xenodiagnosis and PCR. In each experiment five randomly selected colonies from each plate were grown in liquid BSK II medium (containing either kanamycin or streptomycin) and total genomic DNA was isolated as previously described (20). For xenodiagnosis, chemically competent *E. coli* DH5α cells were transformed with genomic DNA and plated on lysogeny broth (3) plates containing kanamycin to select for transformants carrying pBS*rpsL*. DNA from kanamycin-resistant DCSmR/pBS*rpsL* *Borrelia* yielded kanamycin-resistant *E. coli* colonies in all cases (15/15), suggesting that pBS*rpsL* was present, while no colonies were observed using DNA from streptomycin-resistant DCSmR/pBS*rpsL* (0/15).

TABLE 1. Antibiotic selection for the loss of pBS*rpsL*

Strain	Plasmid	% of colonies,[a] compared to no selection, in the presence of:		
		Kanamycin	Kanamycin + streptomycin	Streptomycin
B31-A	pBS*rpsL*	101 (±5.1)	0 (±0)	0 (±0)
DCSmR4	pBSV2	105 (±9.2)	85 (±8.6)	96 (±8.6)
DCSmR4	pBS*rpsL*	106 (±20.0)	0 (±0)	0.04 (±0.01)

[a] Values are means ± standard errors of the means for four independent experiments.

To confirm these results, total genomic DNA was used to screen for the presence of pBS*rpsL* by PCR analysis using primers PflgB5+MfeI and KanR 488R. All 15 kanamycin-resistant DCSmR/pBS*rpsL* clones were positive for pBS*rpsL* by PCR, while all 15 streptomycin-resistant DCSmR/pBS*rpsL* clones were negative. Although the absence of a genetic element cannot be proven, these data, taken together, suggest that streptomycin can be used to select for the loss of the wild-type *rpsL* allele, conferring streptomycin susceptibility, in a merodiploid (DCSmR/pBS*rpsL*). Thus, *rpsL* can function as a counterselectable marker and should be a useful molecular tool for genetic experiments that require the loss of a DNA sequence. We are currently isolating *rpsL* mutations in low-passage infectious strains, and we are attempting to apply the counterselectable marker system for use in the animal model of Lyme borreliosis.

We thank Steve Lodmell and Meghan Lybecker for thoughtful and critical reading of the manuscript; Mathieu Picardeau (who suggested developing *rpsL* as a counterselectable marker), Jon Graham (who performed the statistical analysis of clones), and Meghan Lybecker for valuable discussions; Patty McIntire (Murdock DNA Sequencing Facility) for DNA sequencing; Philip Stewart for providing pBSV2; and Laura Hall for excellent technical assistance.

J.M.D. is a student at Hellgate High School.

This work was supported by a grant from the National Institutes of Health (AI051486) to D.S.S.

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